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Effect-Directed Analysis of Municipal Landfill Soil Reveals Novel Developmental Toxicants in the Zebrafish *Danio rerio*

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Supporting Information

ABSTRACT: Effect-directed analysis (EDA) is an approach used to identify (unknown) contaminants in complex samples which cause toxicity, using a combination of biology and chemistry. The goal of this work was to apply EDA to identify developmental toxicants in soil samples collected from a former municipal landfill site. Soil samples were extracted, fractionated, and tested for developmental effects with an embryotoxicity assay in the zebrafish *Danio rerio*. Gas chromatograph mass selective detection (GC-MSD) chemical screening was used to reveal candidate developmental toxicants in fractions showing effects. In a parallel study, liquid chromatography−hybrid linear ion trap Orbitrap mass spectrometry was also applied to one polar subfraction (Hoogenboom et al. *J. Chromatogr. A* **2009**, *1216*, 510−519). EDA resulted in the identification of a number of previously unknown developmental toxicants, which were confirmed to be present in soil by GC-MS. These included 11H-benzo[b]fluorene, 9-methylacridine, 4-azapyrene, and 2-phenylquinoline, as well as one known developmental toxicant (retene). This work revealed the presence of novel contaminants in the environment that may affect vertebrate development, which are not subject to monitoring or regulation under current soil quality assessment guidelines.

INTRODUCTION

Effect directed analysis (EDA) is a modern approach used to identify substances in complex environmental samples which cause toxicity (reviewed in [1]). EDA is particularly useful because it fills a gap in current soil quality assessment approaches in which only specified priority pollutants are measured and potentially toxic unknown substances can be missed. EDA uses bioassays, which measure biological effects in organisms or in vitro systems, to direct fractionation of complex samples and subsequent chemical analysis, thereby ensuring that chemicals identified with the approach actually cause effects. In our laboratory, EDA has been successfully applied to identify estrogenic chemicals in a number of complex matrices such as sediment and tissues from wild fish.[2−4]

The goal of this work was to apply the EDA approach to reveal chemicals in a complex environmental sample that can cause abnormal vertebrate development. For this purpose, we used soil samples collected from a former municipal landfill site in the south of The Netherlands. This site is currently under investigation as a potential site for bioremediation and reuse for various functions. To study the effects of chemicals on survival and development, we used an embryotoxicity bioassay established in our laboratory in the zebrafish *Danio rerio*.[5] The zebrafish is an excellent model for studying vertebrate development due to its external fertilization, transparent embyos, rapid embryonic developmental cycle and large clutch sizes.[6] It is rapidly becoming a model for human health studies because of its well characterized development and genetics.[7] During embryotoxicity bioassays, we closely observed zebrafish development during early life stages (up to 6 days post fertilization) and focused on specific developmental effects in various dilutions of soil extracts.

In this work, embryotoxicity assays in the zebrafish directed the fractionation and identification of candidate teratogenic chemicals. To this end, we used gas chromatograph mass selective detection (GC-MSD) screening to reveal tentative chemicals in fractions of the soil sample showing specific developmental effects. In a parallel study, liquid chromatography−hybrid linear ion trap Orbitrap mass spectrometry (LC-MS) was applied to tentatively identify chemicals in one polar subfraction of this soil.[8] Toxicity tests in zebrafish embryos revealed a number of compounds in this soil with previously unknown teratogenic properties.

EXPERIMENTAL SECTION

Chemicals. All solvents were of pro analyze (p.a.) quality or better, and purchased from J.T. Baker (Deventer, The Netherlands) or Merck (Darmstadt, Germany) unless stated otherwise. Dichloromethane was purchased from LGC (Middlesex, U.K.). Chemicals purchased following tentative identification (Tables 1 and 2) included 9-methylacridine (>99%, ACROS, Geel, Belgium), 3,6 dimethylphenanthrene (p.a., Riedel de Haan, Seelze, Germany), squalene (≥98%, Sigma, Steinheim, Germany) and PCB 189

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Table 1. Concentrations of Persistent Organic Pollutants ($\mu g \cdot kg^{-1}$ Dry Weight) in Composite Sample of Soil from the Vlagheide Landfill Site

compound	$\mu g \cdot kg^{-1}$	compound	$\mu g \cdot kg^{-1}$
polycyclic aromatic hydrocarbons		organochlorine pesticides	
naphthalene[a]	2000	pentachlorobenzene	0.49
acenaphthylene	<3	hexachlorobenzene	0.56
acenaphthene	3000	heptachlor	<0.2
fluorene	3300	octachlorostyrene	<0.1
phenanthrene[a]	15700	*o,p′*-DDE	<0.2
anthracene[a]	2200	*p,p′*-DDE	21
fluoranthene[a]	13800	*o,p′*-DDD	44
pyrene	8200	*p,p′*-DDD	150
benz[a]anthracene[a]	4700	*o,p′*-DDT	<0.1
chrysene[a]	3700	*p,p′*-DDT	6.1
benzo[b]fluoranthene	3000	α-HCH	0.19
benzo[k]fluoranthene[a]	1700	β-HCH	1.7
benzo[a]pyrene[a]	3200	γ-HCH	0.09
dibenz[a,h]anthracene	360	*cis*-heptachlor epoxide	<0.02
benzo[ghi]perylene[a]	1900	*trans*-heptachlor epoxide	<0.02
indeno[1,2,3-cd]pyrene[a]	2200	dieldrin	11
sum PAHs (10 VROM)	51500	endrin	<0.05
		aldrin	8.9
		telodrin	23
		isodrin	<0
		α-endosulfan	<0.04
		hexachlorobutadiene	0.1
polychlorinated biphenyls			
PCB28	490		
PCB52	160		
PCB101	60		
PCB118	40		
PCB153	40		
PCB138	50		
PCB180	28		
sum PCBs	868		
brominated flame retardants			
BDE28	<0.016	BDE100	0.19
BDE47	0.25	BDE119	<0.016
BDE49	0.043	BDE138	0.24
BDE66	<0.016	BDE153	1.05
BDE71	<0.016	BB153 + BDE154	0.19
BDE75	<0.016	BDE183	0.91
BDE77	<0.017	BDE190	<0.016
BDE85	0.04	HBCD	2.41
BDE99	0.21	me-TBBP-A	0.09

[a] PAH included in sum of 10 PAHs [11]

(p.a., CIL, Andover, MA, U.S.). The chemicals 1-methylpyrene (≥97.0%), 3,5 diamino-1,2,4 triazole (≥98.0%), and 11H-benzo-[b]fluorene (≥98.0%) were purchased from Fluka, Steinheim, Germany. Retene (technical grade), phenanthrene (98%), and 2-phenylquinoline (99%) were purchased from Aldrich, Steinheim, Germany. The azaarenes 2-azapyrene (>99%) and 4-azapyrene (95.2%) were purchased from MRI, Kansas City, MO, U.S. Dr. F. Ariese of the Department of Analytical Chemistry of the VU University Amsterdam kindly provided 2-methylchrysene.

Study Site. The study site was the former Vlagheide municipal waste landfill site, located 10 miles SE of 's-Hertogenbosch, The Netherlands. Soil samples were taken by Haskoning B.V., The Netherlands, in October 2005, at depths varying from 3 to 18 m. Seven soil samples were pooled, sieved (mesh size 250 μm), homogenized and freeze-dried to produce a composite sample of approximately 1 kg dry weight.

Targeted Analysis of Priority Chemicals in Soil Sample. Targeted analysis of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and brominated flame retardants was carried out in order to characterize priority pollutants in the total composite sample, prior to fractionation. The PAHs were analyzed using GC-MSD (Agilent 6890 with an Agilent 5973 network quadropole MSD) in SIM mode by screening the 16 EPA PAHs based on a standard protocol (NIST SRM2260a). The PCB/OCBs were determined with GC-μECD (Agilent 6890) based on a standard protocol (Accustandard, S-1878−2X-4 ML-CLP). The brominated diphenyl ethers (BDEs) were analyzed using a GC-MSD (Agilent 6890 with an Agilent 5973 network quadropole MSD) in NCI mode using a standard BDE mix (BDE-MXE, Wellington Laboratories Inc., Guelph ON, Canada).

Effect-Directed Analysis. *Clean Up and Extraction.* Samples of 10 g were taken from the composite soil sample and subjected to pressurized liquid extraction (PLE) with acetone and dichloromethane (25/75 volume ratio) in an accelerated solvent extraction (ASE) apparatus (Dionex, ASE200, Sunnyvale, CA, USA). The composite soil sample extracts were subjected to gel permeation chromatography (GPC) clean up with dichloromethane. The PLE extraction and GPC procedures are described elsewhere.[2,9] The resulting extracts were dissolved in 1 mL methanol:water (1:1 v/v) and fractionated as described below.

Fractionation. Extracts corresponding to 10 g soil were fractionated in three consecutive series of fractionations, according to a previously described system.[3] Briefly, a reversed phase high pressure liquid chromatography (RP-HPLC) system (Shimadzu, Duisburg, Germany) with a C18 semi preparative column (Vydac 2Tp510, 250 × 10 mm, 5 μm) and a 4.7 $mL \cdot min^{-1}$ water/methanol gradient was used.

Series 1. For the first series of fractionation, the water/methanol gradient started at 50% water: 50% methanol and increased to 100% methanol over a period of 50 min, followed by an extra 40 min at 100% methanol, with fractions collected at five different retention time intervals, from 0 to 10:50 min, 10:50−22:57 min, 22:57−38:05 min, 38:05−63:00 min, and 63:00−90:00 min. A solvent blank was also included in the fractionation series. The resulting fractions were evaporated and taken up in 50 μL methanol.

Series 2. On the basis of the results of zebrafish embryotoxicity tests in the Series 1 fractions, a second round of RP fractionation was carried out between Fraction 2 and Fraction 4 of Series 1. Using the same gradient conditions as described above, 20 fractions were collected with retention time intervals of three minutes, starting at 6 min and ending at 66 min retention time. The resulting Series 2 fractions were evaporated and taken up in 50 μL methanol. The collected fractions were again analyzed for embryotoxicity.

Series 3. A third and final series of fractionation was then performed, in which 1 min subfractions were collected from 18 to

Table 2. Compounds in Composite Landfill Soil Sample Tentatively Identified with Chemical Screening and Concentration ($\mu g \cdot kg^{-1}$ Dry Weight) in Soil[a]

fraction 6a	conc in fraction	fraction 13a	conc in fraction
3,5-diamino-1,2,4-triazole	n.c.	1-methylpyrene	n.c.
9-methylacridine	18	3,6-dimethylphenanthrene	n.c.
2-phenylquinoline	6	2,3,3′,4,4′,5,5′-heptachloro-1,1′-biphenyl (PCB 189)	n.c.
2-azapyrene[b]	n.c.	11H-benzo[b]fluorene	141
4-azapyrene[b]	189	2-methylchrysene	n.c.
		1-methyl-7-(1-methylethyl)-phenanthrene (retene)	83
		squalene	n.c.

[a] n.c.: compound not confirmed in fraction (see also Supporting Information Figure S1). [b] Also identified by LC-MS analysis in separate study [8]

26 min retention time interval, and from 39 to 47 min retention time interval. The 18−26 min retention time interval corresponded with the fractions 5 to 7 in Series 2. The subfractions of 1 min retention time were thus named fraction 5a, 5b, 5c, 6a, 6b, 6c, 7a, 7b, and 7c. The 39−47 min retention time corresponded with fractions 12 to 14 in Series 2. The subfractions of 1 min retention time were thus named fraction 12a, 12b, 12c, 13a, 13b, 13c, 14a, 14b, and 14c. The collected subfractions in the third series were evaporated and taken up in 50 μL methanol. In order to collect sufficient extract for chemical and toxicity analysis, the third series of fractionation was carried out four consecutive times with 10 g soil each, resulting in a total of 200 μL extract per fraction representing 40 g of soil.

Embryotoxicity Assays with Zebrafish. The methanol fractions collected in the three series of fractionations were first transferred to dimethylsulfoxide (DMSO, spectrophotometric grade 99.9%, Acros, Geel, Belgium) for testing in the embryotoxicity tests. For this, volumes of methanol (50 μL) corresponding with 10 g soil were gently evaporated under nitrogen gas and taken up in 25 μL of DMSO. Therefore, 1 μL of DMSO represents the chemical extract from 0.4 g of soil. Embryos were exposed to 5 μL extract per experiment (see below), representing a total exposure to the extract of 2 g of soil.

Zebrafish (*Danio rerio*) are cultured in our laboratory in a flow-through aquarium system (Schwarz, Tübingen, Germany) according to a permit from VU University Animal Welfare Committee. The zebrafish are maintained in a photoperiod of 14 h light, 10 h darkness, and are fed three times daily with either dry fish food (Tetramin, Tetra Werke, Melle, Germany) or live brine shrimp (*Artemia salinas*). Water quality is monitored daily, and is maintained at 26 ± 1 °C, pH 7.5 ± 0.2, and conductivity 400−600 μS. Spawning was induced by separating male and female fish overnight and joining them the next morning in a breeding aquarium containing a mesh net. Eggs were collected and fertilization and quality were assessed under a stereo microscope (Leica model M7.5, Leica Microsystems, Rijswijk, The Netherlands). Exposure medium was prepared by adding extracts or test chemicals dissolved in DMSO at a volume of 5 μL (0.01% solvent) to glass beaker dishes containing 50 mL Dutch Standard Water (DSW: 100 mg L^{-1} $NaHCO_3$, 20 mg L^{-1}, $KHCO_3$, 180 mg L^{-1} $MgSO_4$ and 200 mg L^{-1} $CaCl_2$) at 27 °C. DMSO was incorporated in all experiments as a negative control and in the case of experiments with individual chemicals, 50% of the exposure medium was renewed daily. Embryos were exposed to chemicals in concentrations of 1, 5, 10, and 20 μM. Renewal of the extracts from the fractions was not possible however, due to the limited amount available. Fertilized eggs (25−30) were added to the beakers containing exposure medium within 3 h post fertilization (hpf). The developing zebrafish were scored daily for lethality and developmental malformation during the first 72 h, and then again following 144 h (6 day) exposure. For this, a list of sublethal and teratogenic end points previously described by Nagel, 2002 [10] was used. Images were obtained with a digital camera coupled to a stereomicroscope. Two to three independent replicates of each embryotoxicity experiment were performed.

Chemical Screening. Portions of 1 μL of fractions 6a, 6b, 6c, 13a, 13, and 13c were injected on a GC-MSD (Agilent 6890 with an Agilent 5973 network quadropole Mass Selective Detector) operated in full scan mode (m/z 50−650).[3] Mass spectra were deconvoluted using AMDIS and compared with reference spectra in the National Institute of Standards and Technology (NIST) database (NIST/EPA/NIH Mass Spectral Database, NIST 1992, 1998, Gaithersburg, MD, USA) for tentative identification. Only substances with the highest probability of a match with library compounds based on retention time were included in the list of tentatively identified compounds (Table 2). One polar fraction (6a) was also subjected to liquid chromatography−hybrid linear ion trap Orbitrap mass spectrometry, which was performed in the group of Prof. P. de Voogt at KWR Watercycle Research Institute, The Netherlands. This analysis is described elsewhere.[8]

Confirmation of RP-HPLC Fractionation Pattern and Presence in Soil. A test mix of tentatively identified chemicals in Fractions 6a and 13a (Table 2) was made by diluting concentrated stock solutions in methanol and fractionating the mix according to the third RP-HPLC fractionation as described above. Fractions of 1 min retention time corresponding with Fractions 6a and 13a were collected and injected on the GC-MSD (full scan mode) to confirm the elution of the chemicals in the appropriate fraction. Following the confirmation of RP-HPLC retention pattern of five compounds (11H-benzo[b]fluorene, 9-methylacridine, 4-azapyrene, and 2-phenylquinoline, and retene) in the corresponding fraction, their concentration was determined in the soil extracts using GC-MSD as described above.

RESULTS AND DISCUSSION

Soil from Former Municipal Landfill Contaminated with Persistent Organic Pollutants. Chemical characterization of the composite soil sample from the Vlagheide landfill site revealed that this site is highly polluted, particularly with PAHs (Table 1). The sum of 10 PAHs is 51.5 mg kg^{-1}, which exceeds the intervention value of 40 mg kg^{-1} set by the Dutch Ministry of Ministry of Housing, Spatial Planning and the Environment.[11] The intervention value indicates whether the functional characteristics

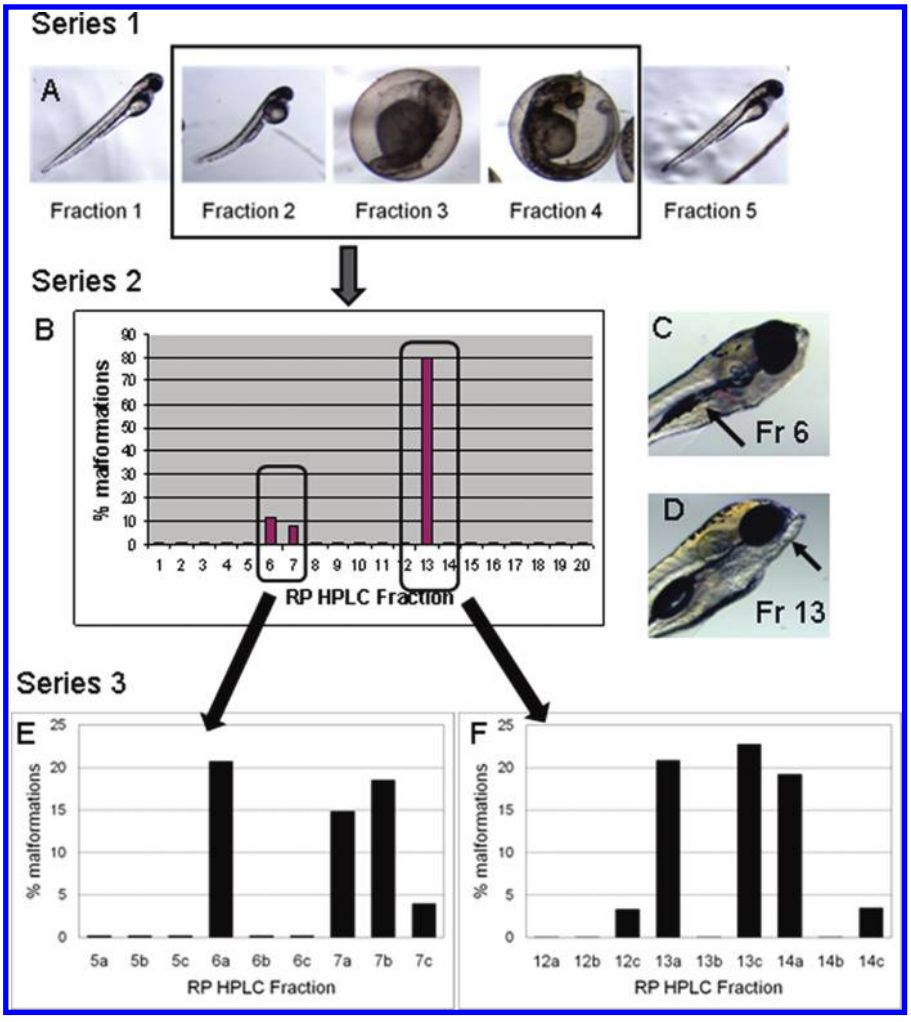
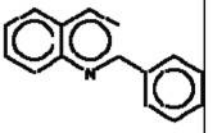
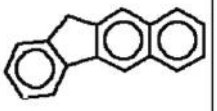
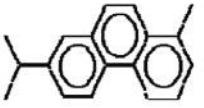
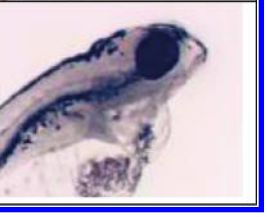


Figure 1. Embryotoxicity in zebrafish embryos exposed to three consecutive fractionation series of a soil extract from a domestic landfill site. (A) First fractionation series: phenotypical effects of five fractions collected using a reversed phase HPLC system with a gradient running from 50:50 methanol: water to 100% methanol for 90 min (see Experimental Section). Fraction 1, and 5: no effects on development after 72 h of exposure, Fractions 2, 3, and 4: notochord (Fraction 2) and lethal (Fraction 3 and 4) effects after 72 h of exposure. (B) Second fractionation series of 20 fractions with 3 min intervals starting at 6 min: percentage of malformed embryos per fraction. (C) Embryos exposed to Fraction 6 show lack of swim bladder inflation (arrow), 144 hpf. (D) Embryos exposed to Fraction 13 show craniofacial malformations (arrow), 144 hfp. (E) Third fractionation series of 1 min intervals from (E) Fractions 5−7 and (F) Fractions 12−14: percentage of malformed embryos. (n = 25−30, each fraction represents the extract from 2 g of soil).

of soil for humans, animals, and plants are threatened, and represent a level of pollution that, if exceeded, would be considered serious soil contamination requiring remediation.[11] Concentrations of PCBs and some organochlorine pesticides were also elevated, with the sum of 7 PCBs close to the intervention value of 1 mg kg^{-1}. The total DDT/DDE values, as well as total drins, exceeded threshold values for healthy soil functioning (0.01 and 0.005 mg kg^{-1}, respectively).[11] The brominated flame retardants, in particular the brominated diphenyl ethers, did not appear to be elevated in this sample. One exception was the brominated flame retardant hexabromocyclododecane (HBCD), with levels of 2.4 $\mu g \cdot kg^{-1}$ approximating levels reported elsewhere in European soils and sediments.[12]

Consecutive Fractionation Leads to Specific Effects on Zebrafish Development. The high level of contamination in this soil, in particular with polycyclic aromatic hydrocarbons such as phenanthrene, pyrene, chrysene, and benz[a]anthracene which are known to cause developmental toxicity,[13,14] led us to hypothesize that exposure to contaminants present in this soil could cause effects on development. Indeed, exposure of zebrafish embryos to a total extract of the original soil sample caused acute lethality (data not shown). The objective of this work was to identify the chemicals with developmental effects in the soil sample. To this end, we performed a series of three consecutive fractionations. Exposure of zebrafish embryos to the first series of five broad fractions resulted in lethal effects on the embryos in Fractions 2, 3, and 4, whereas no effects were found in Fractions 1 and 5 (data not shown). Representative phenotypical effects of exposure to the fractions are depicted in Figure 1A. We therefore performed a second fractionation scheme in this time interval of Fractions 2 to 4 (i.e., 6 to 66 min) in order to further separate this complex mixture. Twenty fractions of 3-min time intervals were collected and exposed to the developing zebrafish. Lethal effects of the fractions were no longer observed for the 3 min fractions, indicating that reducing the complexity of the mixture also reduced overt toxicity. The less complex fractions in Series 2, however, resulted in developmental malformations in embryos exposed to Fractions 6, 7, and 13 (Figure 1B). After 144 h of

Table 3. Chemicals Identified in Landfill Soil with Effect-Directed Analysis, Their Reported Toxicity, And Phenotypical Effects in Zebrafish *Danio rerio* Exposed to 1−5 μM from Fertilization to 144 h Post Fertilization (hpf))

Chemical, Formula, CAS Registry number, Molar weight	Class/ use	Structure	Reported (eco)toxicity	Phenotype, 144 hpf
Fraction 6a				
9-methylacridine $C_{14}H_{11}N$ CAS no. 611-64-3 MW=193.24	N-PAC		Genotoxicity (18)	
4-azapyrene $C_{15}H_9N$ CAS no. 194-03-6 MW=203.24	N-PAC, azaarene		Bacterial mutagenicity (20)	
2-phenylquinoline $C_{15}H_{11}N$ CAS No. 612-96-4 MW=205.25 Conc. in soil: 6 µg.kg^{-1} dry weight	quinoline alkaloid		No ecotox data found	
Fractie 13a				
11H-benzo[b]fluorene $C_{17}H_{12}$ CAS No. 243-17-4 MW=216.28 Conc in soil: 140 µg.kg^{-1} dry weight	PAH		CYP1A inducer (21)	
1-methyl-7-(1-methylethyl)-phenanthrene (retene) $C_{18}H_{18}$ CAS No. 483-65-8 MW=234.34 Conc. in soil: 80 µg.kg^{-1} dry weight	Alkyl-PAH		"Blue-sac disease" inc. cranio-facial abnormalities in fish embryos (22,23)	

exposure to Fraction 6, 11% of embryos in fraction 6 showed malformations such as failure of the swim bladder to inflate (Figure 1C), and tail and notochord malformations. Exposure to fraction 13 resulted in 80% of embryos with craniofacial deformities (Figure 1D), often coupled with heart and/or yolk sac edema. A number of reports have documented effects of environmental chemicals on swim bladder inflation in developing fish embryos, including the PAHs naphthalene, anthracene and chrysene,[13] the pyrethroid pesticide cypermethrin [15] and the organophosphorous pesticide diazinon.[16] Effects on craniofacial development in fish embryos have been reported after exposure to chemicals such as TCDD [17] and complex mixtures of PAHs.[13]

Identification of Developmental Toxicants in Fractions. In order to tentatively identify chemicals in Fractions 6 and 13 that may have caused such specific developmental effects as lack of swim bladder inflation and craniofacial deformities, we screened the fractions with gas chromatograph mass spectrometry screening of these fractions. This resulted in a list of over 100 chemicals (data not shown). We needed to reduce the retention time interval in order to improve the resolution and reduce the number of compounds per fraction. We therefore performed a third series of fractionations, in which we collected subfractions of one-minute retention time intervals around Fractions 6 and 13. As it is difficult to exactly replicate the fractionation process, we collected one-minute subfractions from Fractions 5 to 7, and from Fractions 12 to 14. The one-minute subfractions were then tested in the zebrafish embryotoxicity assay. Compounds present in Fraction 6a, but not 6b or 6c, resulted in 20% of the embryos exhibiting developmental malformations (Figure 1E), which included lack of swim bladder inflation. Exposure to Fractions 13a, 13c and 14a, but not 13b, resulted in about 20% developmental malformations (Figure 1F), which mainly consisted of craniofacial deformities.

In order to reduce the number of candidate chemicals in the active fractions, GC screening was performed in fraction 6a, as well as the "non-toxic" Fractions 6b and 6c, which allowed the elimination of compounds that were present in all three fractions. GC-MS screening was also performed in the active Fractions 13a

and 13c, as well as in the "non-toxic" fraction 13b. In addition, LC-MS screening of fraction 6a was also performed elsewhere, resulting in the identification of a compound with a molecular weight of 203.24, which could be represented by three isomers of the nitrogen polycyclic aromatic compound (PAC) azapyrene.[8] A list of compounds tentatively identified in Fractions 6a and 13b and for which we could obtain standards is shown in Table 2. In order to verify that the compounds were actually present in the fractions causing developmental toxicity, we prepared a test mix containing all candidate chemicals shown in Table 2, and subjected this mix to the RP-HPLC fractionation procedure and GC-MSD analysis to determine if they actually elute in the appropriate fractions. Using this procedure, we confirmed the presence of three chemicals in fraction 6a: 9-methylacridine, 4-azapyrene, and 2-phenylquinoline; and two chemicals in fraction 13a: 11H-benzo[b]fluorene and retene. Several other tentatively identified compounds could not be confirmed, illustrating a drawback of this approach in consistently assigning correct identities to masses. It should be noted, however, that two of these nonconfirmed compounds, 2-azapyrene and 1-methylpyrene, did cause developmental toxicity in zebrafish embryos at 1 μM test concentration (Supporting Information Table S1). It should also be mentioned that we cannot exclude that we may have missed other compounds that may have coeluted in the same peaks as the candidate chemicals shown in Table 2.

Novel Soil Developmental Toxicants Identified with EDA. The chemicals confirmed in fraction 6a included two heterocyclic PACs, 9-methylacridine and 4-azapyrene (Table 3). 9-methylacridine is a metabolite of acridine, a member of the azaarene group of heterocyclic PAHs, which contain one nitrogen atom in one of the aromatic rings.[18] The azaarenes are more water-soluble than the well-known homocyclic PAHs, and are known to cause genotoxicity (reviewed in [18]). Effects of this N-PAC on vertebrate development, however, have not been reported before. Exposure of zebrafish embryos to 5 μM 9-methylacridine resulted in developmental effects such as lack of swim bladder inflation (Table 3), which was a specific phenotypical effect observed in embryos exposed to Fraction 6a. Similar to 9-methylacridine, 4-azapyrene is a nitrogen-containing PAC which has been found in sediment samples [19] and has been reported to cause bacterial mutagenicity.[20] Nothing, however, is known of its potential to induce developmental toxicity. Exposure of zebrafish to 1 μM 4-azapyrene caused mortality and developmental malformations (Table 3). The third chemical confirmed in Fraction 6a, the quinoline alkaloid 2-phenylquinoline, caused similar phenotypical effects as seen by exposure to Fraction 6a, namely lack of swim bladder inflation (Table 3). To our knowledge, nothing has been reported before of possible teratogenic or other toxicological effects of this compound.

Two PACs were confirmed in Fraction 13a, namely 11H-benzo[b]fluorene (BBF) and retene. Exposure of zebrafish embryos to 11H-benzo[b]fluorene, resulted in clear toxic effects, including craniofacial malformations at 1 μM (Table 3). Although developmental toxicity of this alkyl PAC has not been reported before, BBF has been shown to induce CYP1A activity through binding to fish aryl hydrocarbon receptors,[21] a mechanism involved in the embryotoxicity of many PAHs.[13] Exposure to the pyrogenic PAH retene also resulted in effects similar to those found in embryos exposed to the fraction; most embryos showed craniofacial malformations at a concentration of 1 μM (Table 3). Retene is a known inducer of "blue sac disease" in fish embryos, which is characterized by elevated rates of mortality associated with pericardial and yolk sac edema, hemorrhaging, craniofacial deformities, impaired cardiac development, and circulatory failure.[22,23]

Targeted chemical determination of the five identified developmental toxicants also confirmed their presence in the soil, at concentrations ranging from 6 to 189 $\mu g \cdot kg^{-1}$ dry weight (Table 2). To estimate their dissolved concentrations in the exposure medium in the embryotoxicity assays, we calculated expected water concentrations given that the chemicals were dosed in extracts representing 2 g of soil. Although this "worst-case" calculation does not take partitioning into account, it indicates that these chemicals would be present in nominal water concentrations ranging from 0.001 to 0.004 μM (2-phenylquinoline and 9-methylacridine) to 0.02−0.04 μM for 4-azapyrene, 11H-benzo[b]fluorene and retene. In a separate study, detailed concentration−response studies for 4-AP, BBF and retene have been performed in our laboratory, and indicate a no-effect concentration (NOEC) for these chemicals of 0.01 μM (Hawliczek et al., submitted). The calculated nominal water concentrations of these three chemicals from the soil extracts are two to four times above the NOEC, indicating that they may be present at concentrations in the soil that can cause developmental toxicity in zebrafish embryos.

EDA Is a Complementary Approach for Soil Quality Assessment. The presence of these newly identified soil toxicants would have been missed with conventional soil chemical quality assessment, in which the concentrations of a select group of priority pollutants in soils are determined and compared with environmental or human health targets. Importantly, the priority pollutants shown in Table 1 and originally identified with targeted analysis before embarking on the EDA, such as the "standard" homocyclic PAHs and PCBs, did not appear as relevant developmental toxicants in this work. The finding of novel toxicants illustrates the importance of biological testing in determining soil quality. Granted, the approach used in this work may represent a "worst case scenario", with extraction of soil with organic solvents concentrating the chemicals and overestimating their bioavailability in situ. It is not known if these chemicals are taken up by soil organisms, or if they may leach out of soils and enter groundwater where they may form a potential source of exposure to humans and wildlife. Azaarenes such as 9-acridine and quinoline, however, have been detected in aqueous environment in previous studies.[24,25]

This work clearly showed that an effect-directed analysis approach is a powerful tool to identify unknown chemicals in a complex environmental mixture which may cause biological effects. In this work, we revealed chemicals in soil with previously undocumented effects on vertebrate development, namely 9-methylacridine, 4-azapyrene, and 2-phenylquinoline, and 11H-benzo[b]fluorene. Current research is focusing on eliciting the effect concentrations and mechanisms of developmental toxicity of these agents, and their toxicity for soil-based organisms and mammals. As nothing is known of the presence of these compounds in leachates of landfills or soil, studies on the environmental occurrence and exposure are imperative to determine the potential risk of these developmental toxicants to humans and wildlife.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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